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Induction of In Vivo Antipolysaccharide Immunoglobulin Responses to Intact *Streptococcus pneumoniae* Is More Heavily Dependent on Btk-Mediated B-Cell Receptor Signaling than Antiprotein Responses

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The relative role of Btk-dependent B-cell receptor (BCR) signaling in the induction of antipolysaccharide (anti-PS) and antiprotein immunoglobulin (Ig) responses to an intact extracellular bacterium in vivo is unknown. Btk^{low} mice exhibit reduced BCR signaling but largely restore B-cell development. Btk^{low} mice immunized with intact *Streptococcus pneumoniae* elicit reduced anti-PS but normal antiprotein Ig responses. Immunization of Btk^{low} mice with PS-protein conjugate in saline results in an even more profound defect in the anti-PS but not antiprotein response, which is largely restored by use of a CpG-containing oligodeoxynucleotide as an adjuvant. These data demonstrate a greater dependence on Btk-mediated BCR signaling for physiologic anti-PS relative to antiprotein responses, as well as the existence of a compensatory Toll-like-receptor-mediated signaling pathway naturally triggered in response to intact bacterial pathogens.

Bruton's tyrosine kinase (Btk) plays a key role in B-cell receptor (BCR)-mediated signal transduction (3). Btk is critical for the normal development of B-1 and to a lesser extent B-2 B cells. Thus, CBA/N (*xid*) mice (2), which have a loss-of-function point mutation in the Btk gene (29, 42), exhibit a marked reduction in peritoneal B-1a cells, although a more modest decrease in B-1b cells, and a 30 to 50% reduction in splenic B-2 cells, including both marginal zone and follicular subsets (8, 9, 30). *xid* (6, 34) and Btk$^{-/-}$ (15) mice also exhibit marked defects in Ig induction in response to soluble T-cell-independent type 2 (TI-2) antigens (e.g., polysaccharides). In contrast to soluble TI-2 antigens, the TI-1 antigens trinitrophenol (TNP)-lipopolysaccharide and TNP-*Brucella abortus* elicit normal immunoglobulin M (IgM) and IgG2 although reduced IgG3 responses in *xid* mice (24, 38), perhaps reflecting the adjuvant effect of the associated Toll-like receptor (TLR) activity intrinsic to the TI-1 but not TI-2 antigen. Ig responses to T-cell-dependent (TD) antigen, relative to those to TI-2 antigen, are variously and less severely affected in *xid* or Btk$^{-/-}$ mice, with primary responses more defective than those following secondary immunization (4, 13, 15, 26, 33). Nevertheless, Btk appears to function as a BCR signal threshold modulator rather than as an essential component of the BCR signaling pathway (32). Thus, *xid* B cells can respond to particulate TI-2 antigens, such as TNP-sephadex or TNP-polyacrylamide (23). Additionally, defective TI-2 responses in *xid* mice can be corrected by coimmunization with a TLR agonist, such as 8-mercaptoguanosine (1, 21). Finally, TI-2 responses in *xid* mice can be partially reconstituted through provision of T-cell help (7, 18).

Defective humoral immune responses in *xid* or Btk$^{-/-}$ mice could result from a combination of defective B-cell subset development and loss of Btk-mediated BCR signaling in the B cells that are present. In this regard, *xid* mice receiving one allele of a murine Btk transgene driven by the Ig heavy chain promoter and enhancer and expressing 25% of wild-type endogenous levels of Btk restore splenic B-2 cell development to wild-type levels and have a more modest decrease in peritoneal B-1a cells than *xid* mice (31). Nevertheless, these mice still have defective BCR signaling and weaker Ig responses to the soluble TI-2 antigen TNP-Ficoll than wild-type mice. Essentially similar observations were made with *xid* mice containing a transgene encoding the antiapoptotic protein Bcl-2 (43). Since B-1 cells do not participate in the TNP-Ficoll response (10), these data strongly suggest a direct role for Btk-dependent BCR signaling in Ig responses to soluble TI-2 antigens. The latter studies did not evaluate Ig responses to soluble TD antigens, which are also reduced, albeit less dramatically, in *xid* mice.

The studies discussed above collectively indicate that Ig responses, particularly to isolated polysaccharide (PS) antigens in *xid* or Btk$^{-/-}$ mice, can vary dramatically depending upon the presence or absence of adjuvant, T-cell help, and/or antigen particulation and the level of restoration of B-cell subset development. In this regard, intact bacterial pathogens coexpress PS and protein antigens and TLR ligands within a particulate structure. Additionally, we previously demonstrated that IgG anti-PS and antiprotein responses to intact *Streptococcus pneumoniae* were both dependent upon CD4$^+$ T-cell help, B7-dependent costimulation, and CD40-CD40 ligand interactions (14, 44). Thus, the relative role of Btk-dependent BCR signaling in directly regulating anti-PS versus antiprotein

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TABLE 1. B-cell subset development is largely restored in Btk^{low} mice[a]

Cell type and mouse group	Total no. of cells (10^6)	% (No.) of cells in subset				
		FB	MZB	B-1	B-1a	B-1b	B-2
Spleen							
Wild type (CBA/CaJ)	43 ± 2.8	21 ± 1.4 (9.3 ± 1.4)	9.6 ± 0.5 (4.1 ± 0.3)	5.3 ± 0.5 (2.3 ± 0.3)			
xid	37 ± 4.2	9.6 ± 1.1 (3.6 ± 0.8)*	5.0 ± 0.8 (1.9 ± 0.5)*	3.6 ± 0.4 (1.4 ± 0.3)*			
Wild type (BALB/c)	61 ± 4.3	25 ± 1.2 (15 ± 1.6)	4.5 ± 0.4 (2.9 ± 0.4)	2.5 ± 0.2 (1.5 ± 0.1)			
Btk^{low}	59 ± 5.4	23 ± 1.7 (14 ± 1.9)	7.7 ± 0.6 (4.6 ± 0.6)*	2.7 ± 0.1 (1.6 ± 0.1)			
Peritoneum							
Wild type (CBA/CaJ)	3.0 ± 0.6				6.6 ± 0.3 (0.2 ± 0.03)	20 ± 2.6 (0.6 ± 0.1)	3.2 ± 0.4 (0.1 ± 0.05)
xid	1.3 ± 0.3				1.4 ± 0.5 (0.02 ± 0.01)*	6.2 ± 1.8 (0.1 ± 0.04)*	1.1 ± 0.2 (0.01 ± 0.0)*
Wild type (BALB/c)	3.0 ± 0.4				16 ± 1.5 (0.6 ± 0.2)	9.2 ± 0.7 (0.3 ± 0.1)	24 ± 0.4 (0.9 ± 0.1)
Btk^{low}	4.0 ± 0.9				3.6 ± 0.5 (0.2 ± 0.1)*	7.9 ± 1.2 (0.3 ± 0.1)	51 ± 0.6 (2.0 ± 0.4)*

[a] Spleen cell and peritoneal cell suspensions were prepared from CBA/CaHN-Btk^{xid}/J (*xid*) and control CBA/CaJ mice and from Btk^{low} and control BALB/c mice (six mice per group). Cells from each mouse were stained separately as indicated in the text for determination of B-cell subset numbers by flow cytometric analysis. Data are presented as geometric means plus standard errors of the means. Significance: *, $P \leq 0.05$ by Student's *t* test. Comparisons were made between Btk^{low} and BALB/c mice and between CBA/CaHN-Btk^{xid}/J (*xid*) and CBA/CaJ mice.

Ig responses to an intact bacterium in vivo remains an open and important question. In this study we determined PS- and protein-specific IgM and IgG responses to both intact *S. pneumoniae* and soluble TD conjugates of pneumococcal PS and protein antigens in *xid* and Btk^{low} mice. We demonstrate that Btk-dependent signaling plays a significantly greater role in stimulating anti-PS, versus anti-protein, responses to intact *S. pneumoniae* and to soluble pneumococcal conjugate in vivo following restoration of B-cell subset development. The relevance of these data in the context of anti-PS and antiprotein responses following natural pneumococcal infections in infants (28, 37, 40, 41) is discussed below.

Btk^{low} mice are Btk$^{-/-}$ mice carrying a wild-type Btk transgene driven by the Ig heavy chain promoter and enhancer, as described previously (31), and backcrossed six generations onto the BALB/c background. These mice express 25% of endogenous levels of the Btk protein in splenic B cells. BALB/c mice (Jackson Labs, Bar Harbor, ME) were used as controls for Btk^{low} mice. CBA/CaHN-Btk^{xid}/J (*xid*) and control CBA/CaJ mice were also obtained from Jackson Labs.

Splenic and peritoneal B-cell subsets in both *xid* and Btk^{low} mice were enumerated, relative to those in wild-type mice, by flow cytometric analysis (six mice per group; cells from each mouse analyzed separately) (Table 1). For enumeration of marginal zone B (MZB) cells and follicular B (FB) cells, spleen cells were stained with rat IgG2b,κ anti-mouse CD21/CD35-phycoerythrin (PE) (clone 7G6) and rat IgG2a,κ anti-mouse CD23-biotin (clone B3B4) followed by streptavidin-PE-Texas Red. MZB and FB cells were identified as CD21^{high} CD23^{low} and CD21^{intermediate} CD23^{high}, respectively. For enumeration of splenic B-1 cells, spleen cells were stained with rat IgG2a,κ anti-mouse B220-PE (clone RA3-6B2) and rat IgG2a,κ anti-mouse CD5-biotin (clone 53-7.3), followed by streptavidin-PE-Texas Red, and B-1 cells were identified as B220$^+$ CD5$^+$. Peritoneal cells were stained with anti-B220-fluorescein isothiocyanate, rat IgG2b,κ antimouse CD11b-PE (clone M1/70), and anti-CD5-biotin followed by streptavidin-PE-Texas Red. B-1a cells were identified as B220$^+$ CD11b$^+$ CD5$^+$, B-1b cells as B220$^+$ CD11b$^+$ CD5$^-$, and B-2 cells as B220$^+$ CD11b$^-$ CD5$^-$. All reagents were purchased from BD-Pharmingen.

Analysis of spleen cells from *xid* mice demonstrated a significant reduction ($P \leq 0.05$) in the absolute numbers of FB (2.6-fold), MZB (2.2-fold), and B-1 (1.6-fold) cells, relative to wild-type (CBA/CaJ) mice (Table 1). A more profound reduction in peritoneal B-cell subsets was observed in *xid* mice relative to wild-type mice (for B-1a, 10-fold; for B1-b, 6-fold; for B-2, 10-fold). In contrast, spleen cells from Btk^{low} mice exhibited no reductions in the absolute numbers of FB, MZB, or B-1 cells relative to those of wild-type mice, with the numbers of MZB cells actually significantly higher (1.7-fold) in the Btk^{low} mice. Similarly, a partial or complete restoration in the numbers of peritoneal B-cell subsets was observed in Btk^{low} mice (for B-1a, threefold lower [$P < 0.05$]; for B1b, no difference; for B-2, 2.2-fold higher [$P < 0.05$]). Since the total numbers of spleen cells were comparable between *xid* and Btk^{low} mice relative to those of their wild-type counterparts, the absolute numbers of B-cell subsets reflected their relative percent representation (Table 1). Collectively, these data are consistent with those reported by others (15, 31) and confirm the ability

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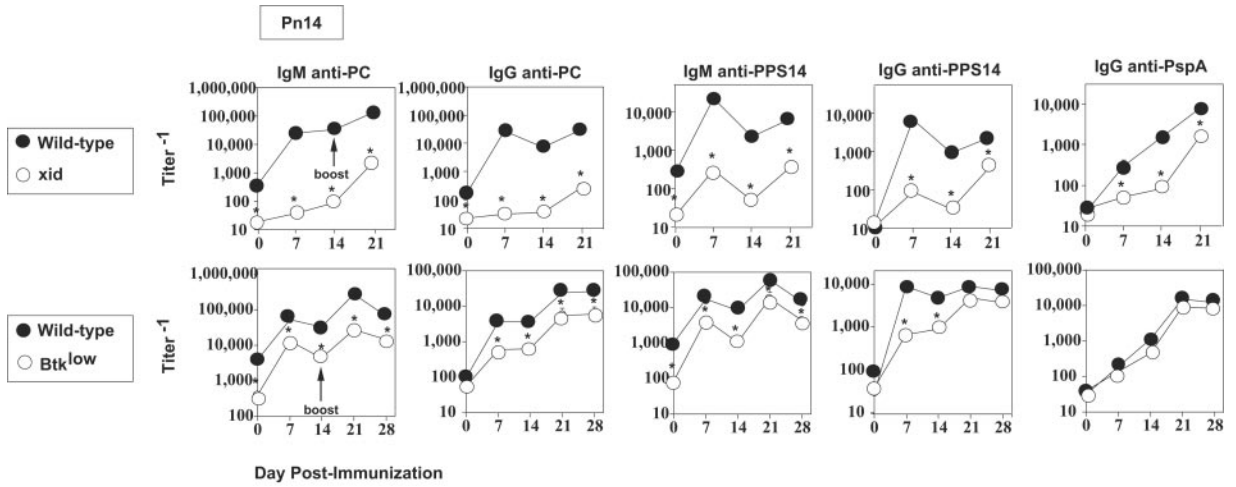

FIG. 1. IgM and IgG anti-PC and anti-PPS14 responses, in contrast to the anti-PspA response, to intact *S. pneumoniae* serotype 14 are selectively defective for Btk^{low} mice relative to those for wild-type mice. CBA/CaHN-Btk^{xid}/J (*xid*) and control CBA/CaJ mice, as well as Btk^{low} and control BALB/c mice (six mice per group), were immunized i.p. with intact heat-killed *S. pneumoniae* serotype 14 (2×10^8 CFU/mouse). Sera were obtained on the indicated days, and antigen-specific IgM and IgG serum titers were determined by enzyme-linked immunosorbent assay. Data are presented as geometric means plus standard errors of the means. Significance: $*$, $P \leq 0.05$ by Student's *t* test.

of the single *btk* transgene to largely restore B-cell subset development in the *xid* mouse.

xid and Btk^{low} mice and their wild-type counterparts were immunized intraperitoneally (i.p.) with heat-killed intact *Streptococcus pneumoniae*, capsular type 14, in saline (2×10^8 CFU *S. pneumoniae* serotype 14/mouse, six mice per group) and boosted in a similar fashion 14 days later. *S. pneumoniae* serotype 14 was prepared, heat killed, and stored, as described previously (14). Serum titers of IgM and IgG specific for the phosphorylcholine (PC) determinant of the cell wall C polysaccharide (C-PS), the capsular PS (PPS14), and the cell wall pneumococcal surface protein A (PspA) were determined on days 0, 7, 14, 21, and/or 28 as described previously (14). Whereas *S. pneumoniae* serotype 14 elicited a strong primary IgM and IgG anti-PC response in wild-type mice, *xid* mice were essentially unresponsive (Fig. 1), consistent with previous reports (22, 27). Secondary immunization with *S. pneumoniae* serotype 14 did result in a detectable IgM and IgG anti-PC response in *xid* mice, but this was still markedly below wild-type levels (for IgM, 56-fold; for IgG, 116-fold). In contrast, Btk^{low} mice elicited detectable, although still significantly reduced, primary IgM (3.9- to 7.5-fold) and IgG (5.8- to 6.1-fold) anti-PC responses relative to those of their wild-type controls. Secondary anti-PC titers for Btk^{low} mice showed reductions relative to titers for wild-type mice similar to those seen for the primary responses. In light of earlier data indicating that the anti-PC response to intact *S. pneumoniae* derives from both B-1 and MZB cells (20), these data suggest that the degree of reduction in anti-PC titers in Btk^{low} mice does not result solely from a reduced representation of responding B-cell subsets. A marked reduction in the primary IgM (46- to 76-fold) and IgG (29- to 75-fold) anti-PPS14 response, as well as the IgG anti-PspA response (>13-fold), was also observed in *xid* mice (Fig. 1). Of interest, secondary immunization of *xid* mice with *S. pneumoniae* serotype 14 resulted in a partial restoration in the IgM (18-fold reduced) and IgG (6.1-fold reduced) anti-PPS14 and IgG anti-PspA (4.6-fold reduced) responses relative to those of wild-type mice. In Btk^{low} mice, the reduction in the primary IgM (3.8- to 8.1-fold) and IgG (5.2- to 13-fold) anti-PPS14 response was significant but more moderate relative to results with *xid* mice, whereas the primary IgG anti-PspA response was comparable to that seen with wild-type mice. Again, secondary immunization of Btk^{low} mice with *S. pneumoniae* serotype 14 resulted in a substantial boost in the IgG, although not IgM, anti-PPS14 response, bringing the IgG titers up to wild-type levels. Comparable boosting of the IgG anti-PspA response was observed in both Btk^{low} and wild-type mice. Collectively, these data demonstrate that the degree of reduction in the anti-PC and anti-PPS14 responses to intact *S. pneumoniae* serotype 14 observed in Btk^{low} mice was significantly out of proportion to any alterations in B-cell subset development. This strongly suggests a role for *btk*-mediated BCR signaling in the responding B cells for induction of an anti-PS response that is greater than that required to elicit an antiprotein Ig response.

As mentioned earlier, both adjuvant (1, 21) and particulation (23) of antigen as is present in intact *S. pneumoniae* serotype 14 can, at least partially, overcome the consequences of defective btk function in B cells. This might potentially obscure an even greater requirement for btk signaling in anti-PS as opposed to antiprotein Ig responses when using soluble antigens in the absence of an adjuvant. To test this hypothesis, we immunized *xid* and Btk^{low} mice and their wild-type counterparts with 1 μg each of soluble PPS14-PspA plus C-PS–PspA conjugates in saline, followed by boosting 14 days later. Serum titers of IgM and IgG anti-PC, anti-PPS14, and anti-PspA were measured on days 0, 7, 14, and 21. The conjugates were synthesized as described by us in detail elsewhere (14). Strikingly, in response to the soluble conjugates in saline, marked and nearly comparable reductions in primary and secondary IgM and IgG anti-PC and anti-PPS14 responses were observed for both *xid* and Btk^{low} mice, relative to results for their wild-type counterparts (Fig. 2), a defect more profound than that observed using intact *S. pneumoniae* serotype 14. In distinct con-

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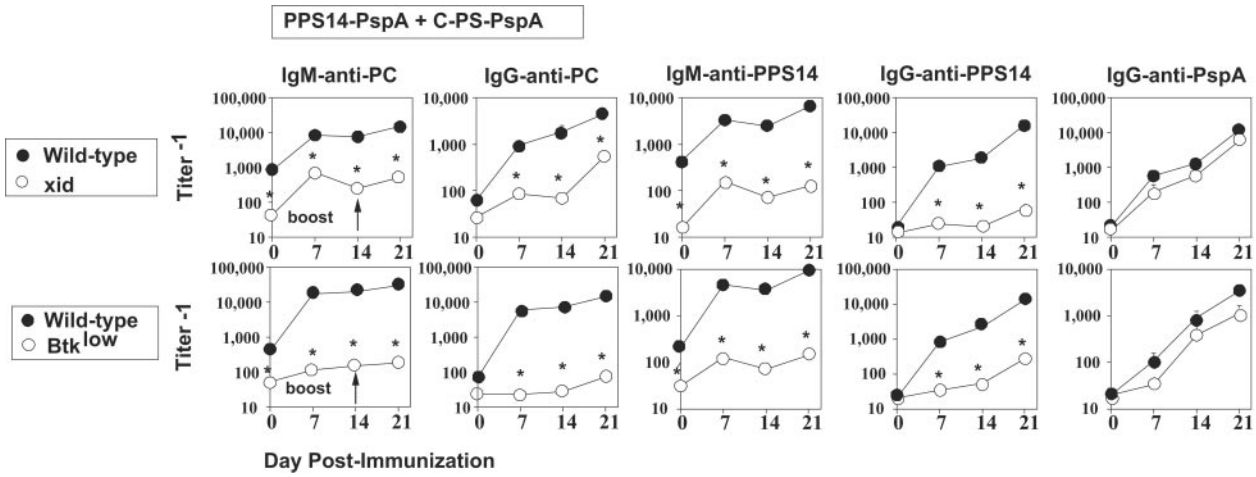

FIG. 2. IgM and IgG anti-PC and anti-PPS14 responses, in contrast to the anti-PspA response, to PPS14-PspA plus C-PS-PspA in saline are selectively defective for Btk^{low} mice relative to those for wild-type mice. CBA/CaHN-Btk^{xid}/J (*xid*) and control CBA/CaJ mice and Btk^{low} and control BALB/c mice (six mice per group) were immunized i.p. with 1 μg each of PPS14-PspA plus C-PS–PspA in saline. Sera were obtained on the indicated days, and antigen-specific IgM and IgG serum titers were determined by enzyme-linked immunosorbent assay. Data are presented as geometric mean plus standard errors of the means. Significance: ∗, $P \leq 0.05$ by Student's *t* test.

trast, the induction of serum titers of IgG anti-PspA for both *xid* and Btk^{low} mice were equivalent to that observed for wild-type mice. Of note, the IgG anti-PC, anti-PPS14, and anti-PspA responses to the soluble conjugates are all $CD4^+$ T cell dependent in wild-type mice (14).

In a final set of experiments, we wished to directly test the notion that the presence of an adjuvant could indeed restore the defective anti-PS responses to the soluble conjugates observed in the Btk^{low} mice and thus at least partially mimic the response to intact *S. pneumoniae* serotype 14. We therefore immunized Btk^{low} and wild-type mice with 1 μg each of soluble PPS14-PspA plus C-PS–PspA conjugates either in saline or adsorbed on 13 μg of alum (Allhydrogel, 2% [Brenntag Biosector, Denmark]) mixed with 25 μg of a 30-mer CpG-containing oligodeoxynucleotide (CpG-ODN) (35), a ligand for TLR9 (12). Mice were boosted in a similar fashion on day 14. Under these conditions, in which a subadjuvant amount of alum is used simply to facilitate the association of the conjugate with the CpG-ODN, the adjuvant activity was almost entirely due to the CpG-ODN (data not shown). In the absence of alum-CpG, the IgM and IgG anti-PC and anti-PPS14, but not IgG anti-PspA, responses were markedly reduced for Btk^{low} mice relative to those for wild-type mice, although partial restoration of IgG anti-PPS14 titers was observed following secondary immunization (Fig. 3), similar to observed results shown in Fig. 2. The addition of alum–CpG-ODN to the conjugates significantly enhanced both IgM and IgG anti-PC, anti-PPS14, and anti-PspA responses in wild-type mice, consistent with several previous reports (5, 16, 17). More remarkably, addition of alum–CpG-ODN almost completely restored the IgM and IgG

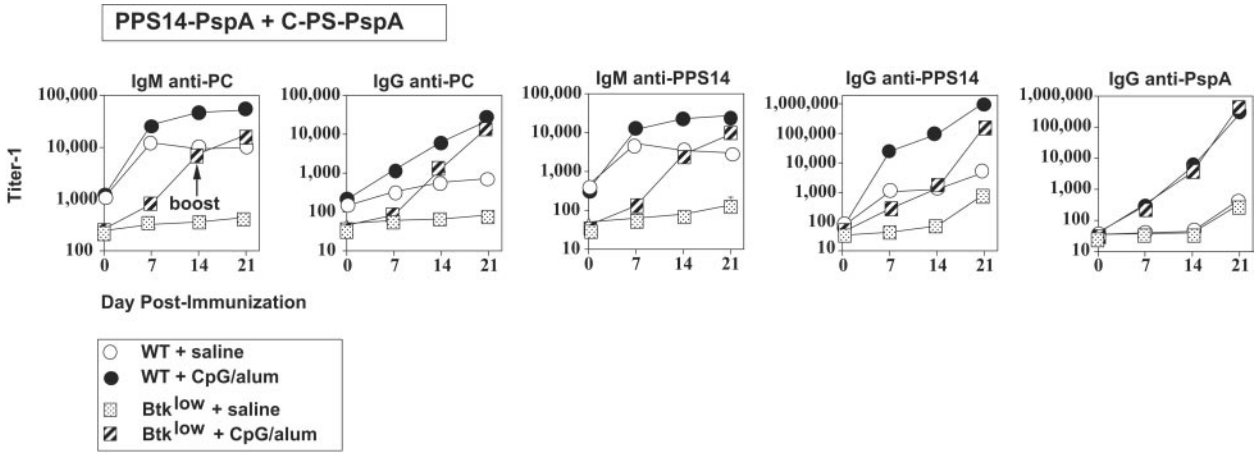

FIG. 3. Alum–CpG-ODN largely restores the IgM and IgG anti-PC and anti-PPS14 response to PPS14-PspA plus C-PS–PspA in Btk^{low} mice. Btk^{low} and control BALB/c mice (six mice per group) were immunized i.p. with 1 μg each of PPS14-PspA plus C-PS-PspA either in saline or adsorbed on 13 μg of alum (Allhydrogel, 2%) mixed with 25 μg of a 30-mer CpG-containing oligodeoxynucleotide (CpG-ODN). Sera were obtained on the indicated days, and antigen-specific IgM and IgG serum titers were determined by enzyme-linked immunosorbent assay. Data are presented as geometric means plus standard errors of the means. Significance: ∗, $P \leq 0.05$ by Student's *t* test.

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anti-PC and anti-PPS14 responses in Btk^{low} mice to wild-type levels, confirming the notion that an adjuvant can overcome defects in Btk signaling for induction of Ig responses (1). The ability of adjuvant to restore anti-PS responses in Btk^{low} mice to the level of those observed in wild-type mice was delayed. Thus, a relatively greater effect of adjuvant was seen on day 14 than on day 7, with optimal effects observed after boosting.

Further analyses of all serum samples obtained in the experiment illustrated in Fig. 3 demonstrated the following (data not shown). (i) The relative primary and secondary serum titers of all IgG isotypes specific for PC, PPS14, or PspA directly mirrored the corresponding serum titers for antigen-specific total IgG. Thus, no selective IgG isotype differences were observed between wild-type and Btk^{low} mice. Further, alum–CpG-ODN enhanced, to similar degrees, the serum titers of all IgG isotypes in wild-type and Btk^{low} mice. (ii). The idiotype of day 21 serum IgM anti-PC antibody in wild-type and Btk^{low} mice in the absence or presence of alum–CpG-ODN was overwhelmingly $T15^{+}$. (iii) The ability of the serum samples to mediate opsonophagocytosis by macrophages in vitro directly mirrored the relative serum titers of PPS14-specific IgG. The opsonophagocytosis assay was performed as described previously (19) with minor modifications.

Collectively, these data strongly suggest that induction of anti-PS responses requires a greater degree of Btk-mediated BCR signaling than induction of antiprotein responses when tested under conditions where both responses are elicited in the presence of $CD4^{+}$ T-cell help, equivalent levels of an adjuvant or its absence, and an antigen form (particulate or soluble) and where B-cell subset development is largely intact. Nevertheless, we show that the presence of a sufficiently strong adjuvant, in this case the TLR9 ligand CpG-ODN, can largely overcome this defect, whereas the degree of natural TLR adjuvant action provided by intact *S. pneumoniae* serotype 14 is only partially effective. Neonatal B cells, like *xid* or Btk^{low} B cells, exhibit defective signaling following BCR cross-linking (11, 36). In this regard, similar to what is observed in *xid* mice, TI-2 responses are also markedly defective in the immature host, whereas T cell-dependent antiprotein responses are less affected (24, 25). Thus, infants respond poorly to isolated PS vaccines but elicit protective anti-PS responses to PS conjugated to an immunogenic carrier protein, which effects recruitment of $CD4^{+}$ T-cell help. As with *xid* B cells, the defective BCR-dependent function of neonatal B cells can be compensated for by TLR-dependent signaling (39). Surprisingly, however, only small minorities of infants less than 2 years of age elicit a detectable systemic or mucosal IgG anticapsular PS response following natural infection with *S. pneumoniae*, whereas a majority induce IgG specific for several *S. pneumoniae* proteins (28, 37, 40, 41). In light of our current data, we propose that this may be partly due to an insufficient level of TLR signaling, provided during natural *S. pneumoniae* infections, in order to overcome the defective BCR signaling that differentially impacts on the anti-PS response in the immature host.

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